The International Journal of Biochemistry & Cell Biology 72 (2016) 1–17

Contents lists available at ScienceDirect

The International Journal of Biochemistry & Cell Biology

journal homepage: www.elsevier.com/locate/biocel

Review article

# New concepts on BARD1: Regulator of BRCA pathways and beyond

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ARTICLE INFO

*Article history:*
Received 16 October 2015
Received in revised form
15 December 2015
Accepted 16 December 2015
Available online 29 December 2015

*Keywords:*
Cancer biomarker
Alternative splicing
Cancer predisposition
Competing endogenous RNA
Cancer treatment

ABSTRACT

For nearly two decades most research on BARD1 was closely linked to research on *BRCA1*, the breast cancer predisposition gene. The co-expression of *BARD1* and *BRCA1* genes in most tissues, the nearly identical phenotype of *Bard1* and *Brca1* knock-out mice, and the fact that BRCA1 and BARD1 proteins form a stable complex, led to the general assumption that BARD1 acts as an accessory to BRCA1. More recent research on both proteins showed that BRCA1 and BARD1 might have common as well as separate functions. This review is an overview of how BARD1 functions and controls BRCA1. It highlights also experimental evidence for dominant negative, tumor promoting, functions of aberrant isoforms of BARD1 that are associated with and drivers of various types of cancer.



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http://dx.doi.org/10.1016/j.biocel.2015.12.008

## 1. BARD1 the major binding partner of BRCA1

In a yeast 2-hybrid screen to identify proteins that associate with the BRCA1 protein *in vivo*, the most promising candidate identified was a novel protein that interacted with the N-terminal region of BRCA1 and which was named **B**RCA1-**A**ssociated **R**ING **D**omain-1, or BARD1 (Wu et al., 1996). The human *BARD1* gene was mapped close to the telomeres of chromosome 2 to 2q34-q35 (Wu et al., 1996), composed of 11 exons, and encoded a protein of 777 amino acids. *BRCA1*, located to chromosome 17, is composed of 24 exons and encodes a 1863 amino-acid protein (Miki et al., 1994). BARD1 has sequence and structural similarities with BRCA1, but not with BRCA2, the second breast cancer susceptibility gene (Wooster et al., 1994) (Fig. 1). BARD1 is specifically homologous to BRCA1 within the conserved RING finger domain at the N-terminus (residues 46–90) (Brzovic et al., 2001a) and the two tandem BRCA1 carboxy-terminal (BRCT) domains at its C-terminus (residues 616–777) (Bork et al., 1997). BRCA1 and BARD1 can form homodimers *via* their RING fingers, but they preferentially form more stable heterodimers, implicating residues 1–109 of BRCA1 and residues 26–119 of BARD1 (Meza et al., 1999), and when hetero-dimerized the RING domains have E3 ubiquitin ligase activity (Brzovic et al., 2001b; Hashizume et al., 2001).

The BRCT repeats are defined by a specific conserved tertiary structure (Glover et al., 2004) and have been identified in many DNA damage repair and cell cycle checkpoint proteins (Callebaut and Mornon, 1997; Huyton et al., 2000). The BRCT modules can form homo/hetero BRCT multi-dimers, BRCT-non-BRCT dimers, and interact with DNA strand breaks (Huyton et al., 2000). Interestingly, mutations in *BRCA1* that cause truncation or loss of both BRCT domains are associated with cancer, suggesting that BRCTs are essential for tumor suppressor functions (Glover et al., 2004; Hayes et al., 2000; Huyton et al., 2000; Williams and Glover, 2003). The BRCT domains of BRCA1 have transcription transactivation activity (Monteiro et al., 1996) and function as phospho-epitope binding domain (Glover et al., 2004; Rodriguez et al., 2003; Yu et al., 2003). In particular it was shown that BRCA1 *via* its BRCT domain binds to the phosphorylated BRCA1-Associated Carboxyl-terminal Helicase (**BACH1**) or also called BRIP1 (Yu et al., 2003). The interaction of BARD1 with these phospho-proteins has only been demonstrated *in vitro* (Thanassoulas et al., 2010). However, the BARD1 BRCT domains interact with a variety of proteins, as discussed below.

In addition to RING and BRCT domains, BARD1 has three ankyrin (ANK) repeats (residues 427-525) located upstream of the BRCT domains, and these are the most conserved region of the BARD1 protein (Ayi et al., 1998; Irminger-Finger et al., 1998) (Fig. 2). A forth, less conserved, ANK repeat was also described (Fox et al., 2008). Varying numbers of ANK repeats are found in many proteins, including Notch, NF-kB, TP53BP, and ankyrins, and mediate protein-protein interactions. The ANK repeats in BARD1 are most homologous with the ANK sequences of TP53BP (Irminger-Finger, unpublished). Several protein interactions have been reported between the BARD1 ANK repeats, including p53 (Feki et al., 2005; Irminger-Finger et al., 2001; Jefford et al., 2004) and NF-κB (Dechend et al., 1999). The combination of RING, ANK, and BRCT domains is a unique feature of BARD1 and not found in any other protein.

Human BARD1 has a nuclear export signal (NES) (residues 102–120) (Rodriguez et al., 2004) and six predicted nuclear localization signals (NLS), situated close to the functional domains (Jefford et al., 2004; Schuchner et al., 2005). NLS and NES are critical for proper intracellular localization of BARD1 and BRCA1, thus affecting their functions.

BARD1 orthologues, beside mouse (Ayi et al., 1998; Irminger-Finger et al., 1998) and rat (Gratas et al., 2001), were reported for *Xenopus laevis* (Joukov et al., 2001), *Caenorhabditis elegans* (Boulton et al., 2004), and *Arabidopsis thaliana* (Lafarge and Montane, 2003) and showed conservation of sequence and exons structure (Fig. 2). Interestingly, the sequence of *BARD1 Canis lupus* exon 1 lacks any similarity with other known BARD1 sequences and encodes an incomplete RING finger (Irminger-Finger unpublished). Sequence data of more than 20 species corroborate the evolutionary conservation of BARD1, but less so for the 5′exons, encoding the BRCA1 interaction (Fig. 2).

## 2. Similar phenotypes of BARD1 and BRCA1 gene knock-outs

The knockout of *Brca1* or *Brca2* genes in mice led to embryonic lethality (Gowen et al., 1996; Hakem et al., 1996, 1998; Ludwig et al., 1997). Similarly, the Bard1-null mouse embryos died between embryonic days E7.5 and E8.5 and showed cell proliferation defects and genomic instability (McCarthy et al., 2003). Partial rescue was obtained in Bard1−/−;p53−/− double knockout embryos, which survived until day E9.5. The Bard1−/−;p53−/− cells displayed an increase of structural and numerical chromosome aberrations compared to p53−/− cells. The phenotypes of *Bard1* knockout mice demonstrated that BARD1 is essential for cell viability and maintenance of genome integrity. It remained unexplained why the depletion of BARD1 leads to lethality only after eight days of embryonic development. Interestingly, *Bard1*, as well as *Brca1* and *Brca2* knockout embryos die at the time when *Bard1*, but not *Brca1*, expression is maximal, which suggests a complex system of mutual regulation of gene expression (Irminger-Finger et al., 1998; Joukov et al., 2001).

Mice with conditional Cre recombination-dependent d of either *Bard1* or *Brca1* in mammary epithelial cells developed breast cancers that resembled the human triple negative breast cancer phenotype seen in carriers of *BRCA1* mutations (Shakya et al., 2008). However, *Bard1* as well as *Bard1/Brca1* double mutants showed both faster initiation of breast cancers than *Brca1* mutants, suggesting a dominant role of BARD1.

Northern blot experiments showed that murine *Bard1* mRNAs were only expressed in spleen and testis (Ayi et al., 1998). More sensitive RNase protection experiments showed expression in most proliferating tissues of the mouse and most elevated in testis and spleen (Irminger-Finger et al., 1998). *Bard1* was also highly expressed during embryogenesis, with a maximum at day 11 (Irminger-Finger et al., 1998), consistent with the embryonic lethality phenotype. More complete expression data are available at *Gene Expression website* ASAP II or GeneCards.org.

During embryogenesis, *Bard1* and *Brca1* transcripts were coordinately expressed until embryonic day 11 and are differentially expressed thereafter and in hormonally controlled organs of adult female mice (Irminger-Finger et al., 1998). In testis, *Bard1* was expressed at all stages of spermatogenesis, whereas *Brca1*

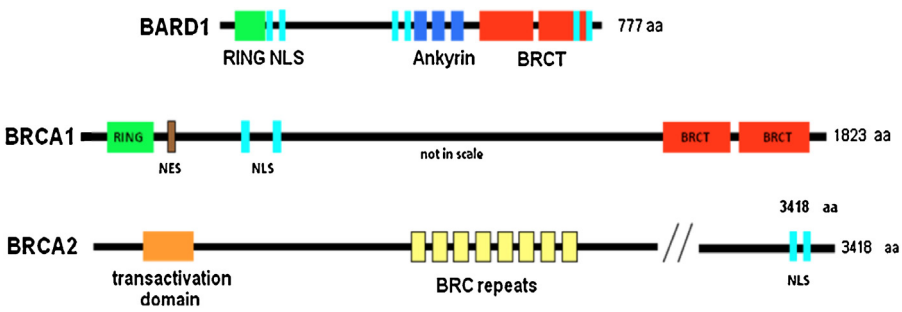


**Fig. 1.** Comparison of protein structures of BARD1, BRCA1 and BRCA2. RING (green), ankyrin (blue), BRCT (red) domains, nuclear export signal (NES, brown), and potential nuclear localization signals (NLS, light blue) are indicated. The third NLS (at amino acid reside 321) of BARD1 is most important for nuclear localization of BARD1. BRCA2 is completely unrelated to either BARD1 or BRCA1 with conserved transactivation domain (TD) and 8 copies of a 70 amino acid motif called the BRC repeats. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

expression was only seen in meiotic and early round spermatocytes (Feki et al., 2004; Scully et al., 1997). These observations were first indications of common and separate functions of *BARD1* and *BRCA1*.

More recent work showed that rather the BRCA1 C-terminus than the RING finger is important for tumor suppression (Shakya et al., 2011). The absence of E3 ligase activity, specified by the BRCA1 and BARD1 RING fingers, did not lead to tumor formation in mice, but mutations in the BRCA1 BRCT domain induced tumors (Shakya et al., 2011).

Similarly, alleles of the *BARD1* homologue of *Arabidopsis* lacking N-terminal domains showed no phenotype, while the deletion of the C-terminal domain led to severe defects of the shoot apical meristem (SAM) (Reidt et al., 2006). As SAM is the stem cell organizing center in plants (Han et al., 2008), these analyses demonstrated a function of *BARD1* in regulating the positioning of stem cells and maintenance of SAM.

## 3. Cellular functions of BARD1 and BRCA1

BARD1 and BRCA1 proteins are synthesized during the S phase of the cell cycle (Hayami et al., 2005) and co-localize with the repair protein Rad51 in nuclear dots (Jin et al., 1997). This co-localization of BARD1 and BRCA1 with Rad51 at sites of DNA damage suggested a function of the BARD1-BRCA1 heterodimer in DNA repair (Stark et al., 2004; Westermark et al., 2003) and triggered research investigating BRCA1 and BARD1 DNA repair functions.

However, BARD1 is also required for normal cell viability, as demonstrated with the *Bard1* knockout mice (McCarthy et al., 2003). *In vitro* repression experiments showed that BARD1 deficiency leads to defects in S-phase progression, loss of contact inhibition of growth, and genetic instability (Irminger-Finger et al., 1998).

BARD1 has specific roles in cell cycle progression, with BRCA1 and distinct from BRCA1. BARD1 plays a chaperone role for BRCA1 translocation into (Fabbro et al., 2002) and retention in the nucleus (Brzovic et al., 2001a; Fabbro et al., 2002; Schuchner et al., 2005), as BRCA1-BARD1 hetero-dimerization masks the NES of both proteins, causing their nuclear retention of the BRCA1-BARD1 complex (Jefford et al., 2004; Rodriguez et al., 2004).

During the cell cycle, at onset of mitosis, BRCA1 controls centrosome duplication (Hsu and White, 1998; Starita et al., 2004; Xu et al., 1999). The localization of BRCA1 to the centrosome depends on BARD1 and the Ran GTPase (Joukov et al., 2006), and centrosome duplication control depends on phosphorylation of BRCA1 by the Aurora A kinase and OLA1 (Brodie and Henderson, 2012a; Matsuzawa et al., 2014; Ouchi et al., 2004; Sankaran et al., 2007). The function of BARD1-BRCA1 heterodimer in the regulation of centrosome amplification requires the nuclear export of BRCA1, promoted by a nuclear export receptor and competed for by BARD1 (Brodie and Henderson, 2012b; Brodie et al., 2012). At later stages of mitosis it is BARD1 is essential for the completion of cytokinesis by dissociating from BRCA1 and interacting with BRCA2 and Aurora kinase B, independently of BRCA1 (Ryser et al., 2009).

A cytoplasmic localization of BARD1 was associated with its apoptotic function and observed after the proteolytic cleavage of BARD1 (Gautier et al., 2000; Jefford et al., 2004; Rodriguez et al., 2004). Thus there is a dynamic interplay of BARD1 and BRCA1 in translocation to DNA repair sites and in shuttling in and out of the nucleus.

BRCA1 and BARD1 have also functions in the regulation of chromatin condensation. In particular they interact with the inactive X chromosome (Xi)-specific transcript (**XIST**) RNA, a non-coding RNA known to coat Xi and to participate in the initiation of its inactivation during early embryogenesis (Ganesan et al., 2004). Female somatic cells lacking wild-type BRCA1, or BRCA1-depleted cells, cannot localize XIST RNA to Xi, but the exogenous expression of wild-type BRCA1 can correct this defect. However, BRCA1-deficient breast cancer cell lines did not show changes of XIST RNA concentration on Xi (Silver et al., 2007; Xiao et al., 2007). Despite these controversial observations, BRCA1 and BARD1 may play a role in the regulation of XIST localization and concentration on Xi and in maintaining heterochromatin structure or function.

These specific individual functions of BRCA1 and BARD1, their interactions with various proteins (Fig. 3), as well as the dissociation of the heterodimer, might be regulated by posttranslational protein modifications such as phosphorylation, ubiquitination, or parsylation.

## 4. The BARD1-BRCA1 heterodimer is an E3 ubiquitin ligase with multiple functions

Better understanding of BRCA1 and BARD1 functions as heterodimer, came with the discovery of the E3 ubiquitin ligase activity of the BRCA1-BARD1 heterodimer (Hashizume et al., 2001). Ubiquitin ligases initiate polyubiquitination, which marks proteins for degradation by the proteasome. Mutations in the BRCA1 RING domain that disrupt the E3 ubiquitin ligase activity of BRCA1-BARD1 are predisposing to breast and ovarian cancer (Brzovic et al., 2001a; Hashizume et al., 2001; Ruffner et al., 2001).

Polyubiquitin chains are commonly K48-linked. The BRCA1–BARD1 heterodimer directs ubiquitin polymerization through K6 linkage on various proteins, including auto-ubiquitination of BARD1 and BRCA1 (Chen et al., 2002; Wu-Baer et al., 2003) (Table 1). However, auto-ubiquitination does not result in degradation of BARD1 or BRCA1, but in an increase of ubiquitin ligase activity and stability of BARD1-BRCA1 (Chen et al., 2002; Mallery et al., 2002; Wu-Baer et al., 2003) and increased DNA damage response (Sankaran et al., 2006).

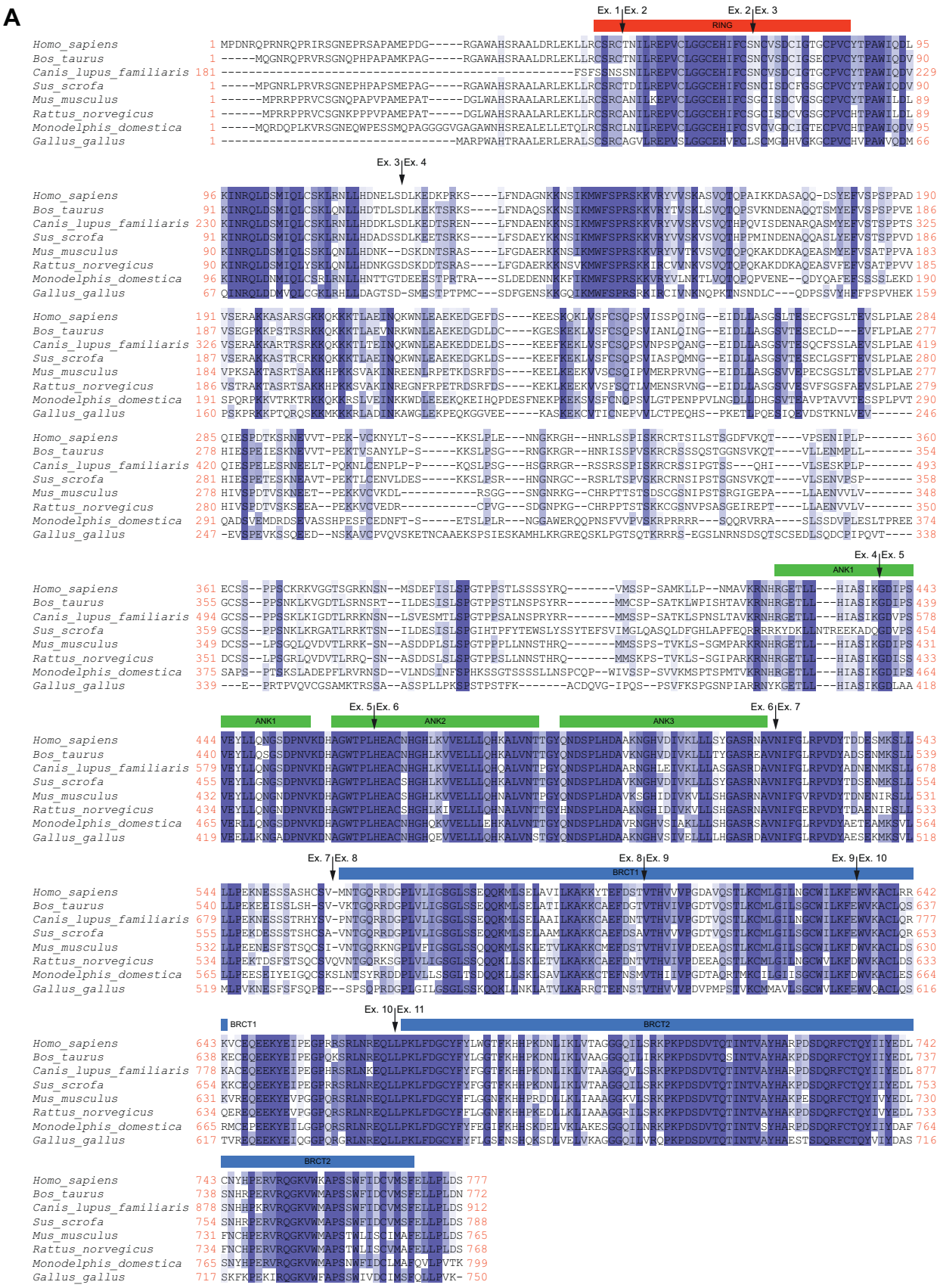


**Fig. 2.** BARD1 is conserved between species. The human BARD1 protein (hBARD1) sequence was aligned with the BARD1 proteins of different species using Clustal Omega multiple sequence alignment tool (http://www.ebi.ac.uk/Tools/msa/clustalo/), and visual representation was done using Jalview software (www.jalview.com). The alignments are color coded in shades of blue for the percentage identity. The exon junctions of hBARD1 are indicated with arrows. The functional domains of BARD1 were defined with the use of the SMART motifs search web tool (http://smart.embl-heidelberg.de/), indicated with overlaid bars: RING (red), ANK (green), BRCT (blue). Human BARD1 aligned with mammalian A) and other (B-D) species. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

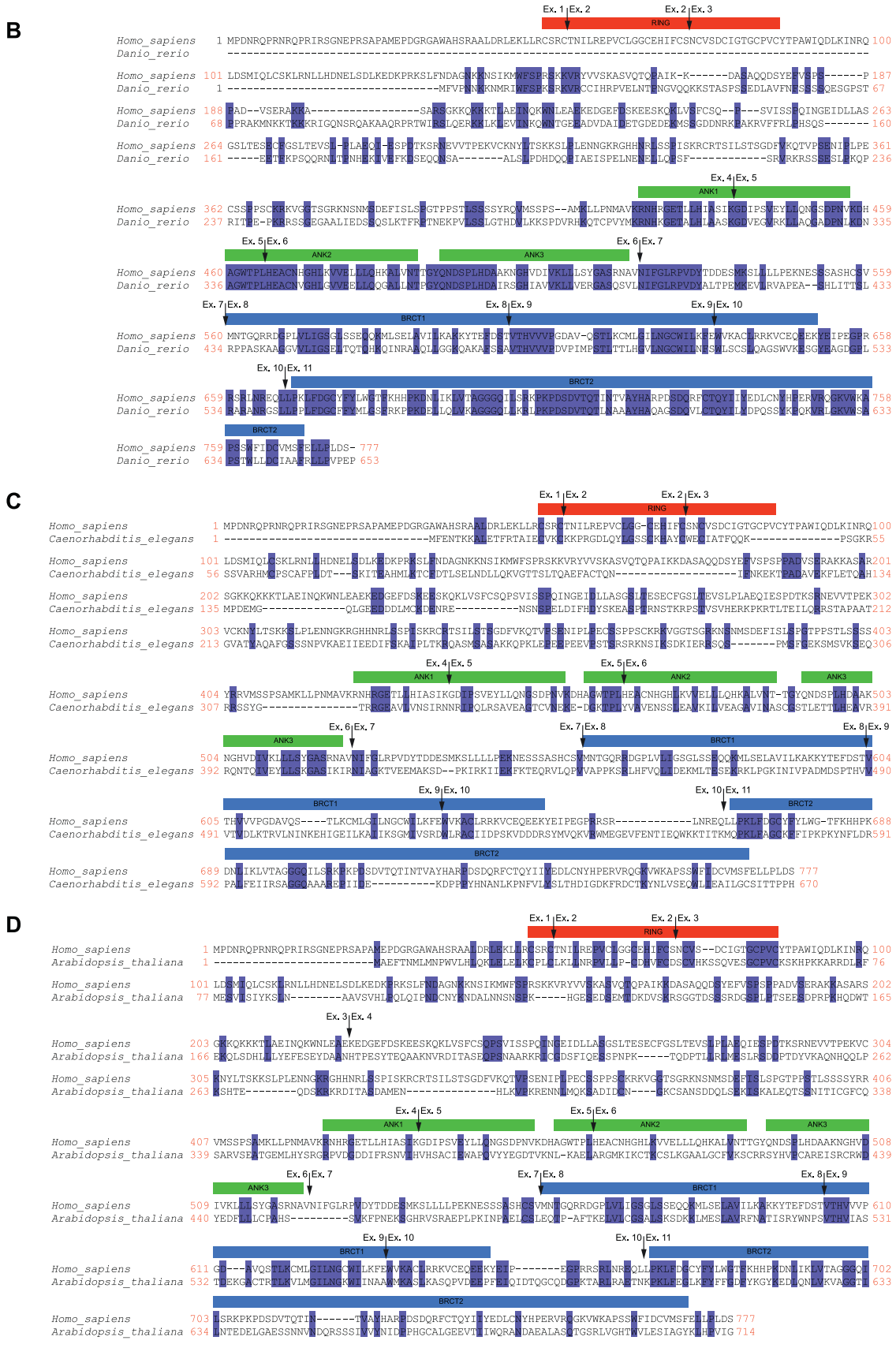


**Fig. 2.** (*Continued*)

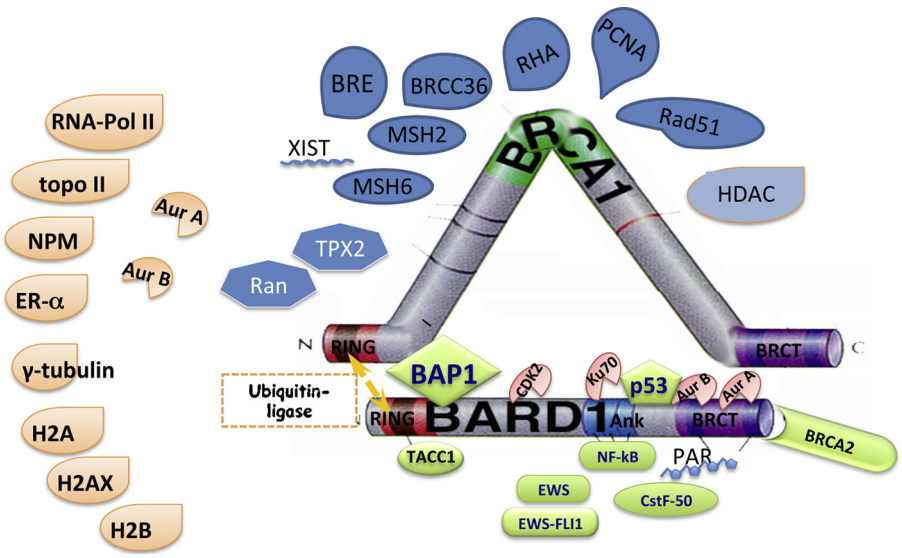


**Fig. 3.** Proteins interacting with the BARD1-BRCA1 complex or BARD1. BRCA1 and BARD1 are shown with RING, BRCT, and ANK domains indicated. BARD1 has been shown to interact with a number of proteins in its role as E3 ubiquitin ligase (orange), as BRCA1-BARD1 complex (blue), or in a BRCA1- independent manner (green) with approximate region of interaction. BAP1 binds to BRCA1 and BARD1 and inhibits ubiquitin ligase activity. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Heterodimer formation and ubiquitination are positive regulators of the BRCA1-BARD1 ubiquitin ligase activity, while phosphorylation of BARD1 by CDK2 and CDK1 on its NH2 terminus completely abolishes the ubiquitin ligase activity (Hayami et al., 2005). The BRCA1 associated protein 1 (BAP1), a de-ubiquitinating enzyme (DUB), also inhibits the E3 ligase activity of BRCA1-BARD1 (Nishikawa et al., 2009). BAP1 interacts with the RING finger of BRCA1 and BARD1 and functions in growth control (Jensen et al., 1998; Mallery et al., 2002; Nishikawa et al., 2009).

A number of potential substrates of the BARD1-BRCA1 E3 ubiquitin ligase were discovered by a quantitative approach (Song et al., 2011), but have not been functionally tested.

### *4.1. BARD1-BRCA1 E3 ligase activity in the DNA damage response pathway*

The dissection of DNA repair pathways showed a major role for the BRCA1-BARD1 heterodimer in homologous repair (Stark et al., 2004). The biochemically defined cellular complex associated with DNA damage response and retaining E3 ubiquitin ligase activity, contained BRCA1, BRCA2, BARD1, and RAD51, as well as BRE and BRCC36, all essential for E3 ligase activity and DNA repair following DNA damage, and was named the *BRCA1-BRCA2 containing complex* (BRCC) (Dong et al., 2003).

BRCA1 and BARD1 also interact with the DNA mismatch repair (MMR) genes, known as predisposition genes for colon cancer (Rustgi, 2007), and bind to MSH2 and MSH6 *in vitro* and *in vivo* (Wang et al., 2001) and might act as downstream effectors of the MSH2-MSH6 complex in DNA mismatch repair signaling.

The BRCA1-BARD1 complex has a role in repair of double strand breaks (DSBs) by regulating the activity of topoisomerase II α (topo II α) in an ubiquitination-dependent manner (Shinagawa et al., 2008; Sordet et al., 2008). Ubiquitination of topo II α by BRCA1-BARD1 was specifically induced by hypoxia, which also upregulates BARD1 expression (Irminger-Finger et al., 2001; Li et al., 2007a). Many cancer drugs are designed to inhibit topisomerase I or II, and deficiencies of BRCA1 or BARD1 might enhance the efficacy of this treatment.

It was shown that BRCA1-BARD1 was responsible for ubiquitination of phosphorylated RNA Pol II in response to DNA damage

**Table 1**
Targets of the BARD1-BRCA1 ubiquitin ligase.

| Target | Linkage | Effect | Function | Reference |
|---|---|---|---|---|
| Histone H2A<br>Histone H2AX<br>Histone H2B | Monoubi. | Stabilization | Chromatin modification | (Calvo and Beato, 2011; Chen et al., 2002; Mallery et al., 2002; Thakar et al., 2010; Xia et al., 2003) |
| RNA polymerase II | Polyubi. | Degradation | Inhibition of transcription | (Kleiman et al., 2005; Starita et al., 2005) |
| Topisomerase II | ND | Modified | Helicase function in repair | (Shinagawa et al., 2008) |
| Nuleophosmin NPM/B23 | Monoubi. | Stability | Apoptosis control | (Sato et al., 2004) |
| γ-tubulin | Polyubi. | Degradation | Microtubule nucleation, centrosome amplification | (Sankaran et al., 2005; Starita et al., 2004) |
| TPX2 | ND | | Spindle orientation | (Joukov et al., 2006) |
| Aurora A | Polyubi. | Degradation | Regulator of mitosis/anaphase | (Bosse et al., 2012) |
| Aurora B | Polyubi. | Degradation | Regulator of mitosis/cytokinesis | (Bosse et al., 2012; Ryser et al., 2009) |
| Estrogen receptor α | Polyubi. | Degradation | Regulation ER response genes | (Dizin and Irminger-Finger, 2010; Eakin et al., 2007) |
| Progesterone Receptor | Polyubi. | Degradation | Regulation of PR response genes | (Calvo and Beato, 2011) |
| BRCA1<br>BARD1 | Monoubi. | Stability | Stability, increase activity | (Chen et al., 2002; Choudhury et al., 2004; Hayami et al., 2005; Wu-Baer et al., 2003) |

(Kleiman et al., 2005; Starita et al., 2005), a critical step in a genome surveillance pathway. Depletion of BRCA1 or BARD1 reduced the ubiquitination of RNA Pol II after DNA damage, and over-expression of BRCA1 in cells stimulated the ubiquitination of RNA Pol II and recovery of cells after exposure to DNA damage (Sankaran et al., 2005). These findings implicate that the BARD1-BRCA1 heterodimer is involved in the regulation of transcription in response to DNA damage.

### 4.2. BARD1-BRCA1 E3 ligase function in modulating chromatin structure

The BRCA1-BARD1 ubiquitin ligase also targets the nucleosome core histones, and the histone variant H2AX. BRCA1 and BARD1 stimulate monoubiquitination of H2A/H2AX *in vitro* (Chen et al., 2002; Mallery et al., 2002; Xia et al., 2003) as well as H2A and H2B (Thakar et al., 2010), which leads to alteration of chromatin structure and opens DNA for transcriptional activity. This result raises the possibility that BRCA1-BARD1 can directly affect nucleosomal structure, dynamics, and function through its ability to modify nucleosomal histones and chromatin structure.

### 4.3. BARD1-BRCA1 E3 ligase function in cell cycle regulation

The regulation of mitosis from onset to cytokinesis is critical in addition to the G1/S and G2/M checkpoints. The duplication and number of centrosomes is important for spindle formation and for maintaining chromosomal stability and ploidy. BRCA1 localizes to the centrosome during mitosis and functions in centrosome amplification (Hsu and White, 1998; Xu et al., 1999). The BRCA1-BARD1 ubiquitin ligase directly regulates centrosome number by targeting γ-tubulin, which is important for the nucleation of microtubule polymerization (Sankaran et al., 2005; Starita et al., 2004).

The BRCA1 N-terminus and BARD1 C-terminus interact with OLA1, an ATPase 1, which also binds to γ-tubulin, and functional studies suggest that is essential for the BARD1-BRCA1 function on γ-tubulin and spindle formation (Matsuzawa et al., 2014).

The nucleolar phosphoprotein nucleophosmin (NPM), or B23, a mitotic protein was identified in a screen for ubiquitin ligase targets of the BRCA1-BARD1 ubiquitin ligase. NPM/B23 is a chaperon protein involved in centrosome duplication and cell proliferation. NPM/B23 is stabilized by ubiquitination (Sato et al., 2004), which is inhibited by BAP1 (Nishikawa et al., 2009). Mutations of NPM/B23 are specifically frequent and predictive markers in hematological disorders (Yohe, 2015).

The BRCA1-BARD1 E3 ligase has functions in mitotic spindle assembly by accumulating the microtubule binding protein TPX2, a spindle organizer, on spindle poles (Joukov et al., 2006). The localization of the BRCA1-BARD1 complex depends on Ran, a GTPase necessary for nuclear import and export, and leads to local TPX2 accumulation. TPX2 is an activator of the Aurora A kinase (Tsai et al., 2003), which is required for centrosome amplification, presumably through the phosphorylation of BRCA1 (Ouchi et al., 2004).

BRCA1 and BARD1 are required for ubiquitination and degradation of the Aurora B kinase in cytokinesis (Ryser et al., 2009). During the formation of the contractile ring around the midbody, Aurora B is gradually degraded and becomes confined to the midbody (Delaval et al., 2004; Ryser et al., 2009). This localization depends on the interaction with the microtubule binding protein TACC1 (Delaval et al., 2004). The TACC1 protein also interacts with BARD1 (Boulton et al., 2004). The abscission of the microtubule bridge linking the two daughter cells at cytokinesis depends on TACC1 and Aurora B (Delaval et al., 2004), BRCA2 (Daniels et al., 2004), and BARD1 (Ryser et al., 2009).

### 4.4. BRCA1-BARD1 E3 ligase function in hormone signaling

*BRCA1* and *BARD1* were first associated with breast cancer, as mutation carriers are at high risk to develop breast (or ovarian) cancers. The biggest risk factor for breast cancer is estrogen, and estrogen deprivation is the preventive measure for carriers of *BRCA1* or *BARD1* mutations. Estrogen acts *via* the estrogen receptors alpha (**ERα**) and beta in transcription activation of genes with pro-proliferative functions.

**ERα** has been identified as a putative substrate for the BRCA1-BARD1 ubiquitin ligase *in vitro* (Eakin et al., 2007). Modulation of BRCA1 or BARD1 levels demonstrated that the BRCA1-BARD1 complex plays a role in ERα ubiquitination and degradation *in vivo* (Dizin and Irminger-Finger, 2010). The ERα-interacting region lies within the BARD1 C-terminus, which suggests that the BARD1 C-terminus might have a function in target recognition. Estrogen treatment induces *BRCA1* and *BARD1* transcriptional upregulation in an ERα-dependent manner, forming a positive feedback loop by which the BRCA1-BARD1 ubiquitin ligase controls cellular levels of ERα (Dizin and Irminger-Finger, 2010). This work provided a mechanism that explains the causative link between estrogen exposure and breast or ovarian cancer. Interestingly ERα ubiquitination and degradation is enhanced by FOXK2 interaction with BARD1 (Liu et al., 2015).

BARD1 and BRCA1 also play a role in progesterone receptor (PR) degradation in the absence of hormone (Calvo and Beato, 2011). Through this function, BRCA1 and BARD1 affect PR recruitment to target gene promoters and transcription. The BRCA1-BARD1 complex interacts and translocates with PR to the hormone-responsive regions of PR target genes and acts in mono-ubiquitination of histone H2A and thus contributes to epigenetic silencing of the respective promoters. Thus, the BRCA1-BARD1 heterodimer regulates transcription of PR response genes in a hormone-dependent and independent manner.

## 5. BRCA1-independent BARD1 interactions and pathways

### 5.1. Inhibition of mRNA maturation

An important finding was BARD1's interaction with the mRNA polyadenylation factor CstF-50 (cleavage stimulation factor), a protein complex involved in the polyadenylation and 3′end cleavage of mRNA precursors (Kleiman and Manley, 1999). The BARD1-CstF-50 interaction was induced by DNA damage and inhibited polyadenylation *in vitro*. A BARD1 mutation Q564H, associated with breast and ovarian cancers, resulted in reduced binding to CstF-50 and diminished inhibition of polyadenylation (Kleiman and Manley, 2001). The CstF-50 binding site on BARD1 is located within the ANK repeats and the BRCT domains of BARD1 (Kleiman and Manley, 1999). The interaction of BARD1 with CstF-50 is regulated by phosphorylation of BARD1 at Thr(714) *in vivo* by ataxia-telangiectasia (ATM), a DNA damage-induced kinase (Kim et al., 2006). These results place BARD1 in a pathway from genotoxic signaling to inhibition of mRNA maturation.

### 5.2. A role in p53 stability and apoptosis

The BARD1 C-terminal half is a module for various interactions (Fig. 3). BARD1 binding to CstF-50 was shown to be competed for by the tumor suppressor p53 (Nazeer et al., 2011), which also binds to the BARD1 ANK repeats and the region between the ANK and BRCT domains (Jefford et al., 2004). Genotoxic stress caused BARD1 mRNA and protein upregulation. BARD1 was found to bind and stabilize p53 and induce apoptosis, but BRCA1 antagonized BARD1-induced apoptosis (Feki et al., 2005; Irminger-Finger et al., 2001).

Several reports addressed the complexity of the BARD1-p53 axis, in particular the aspect of BARD1 or BRCA1 interaction with p53 (Fabbro et al., 2004a, 2004b; Jiang et al., 2011; Rodriguez et al., 2004; Tembe and Henderson, 2007b).

### 5.3. *BARD1 involvement with pathways of oncogenesis*

A specific role for BARD1 in tumorigenesis might exist in cervical cancer. Some strains of the human papillomaviruses (HPVs) are the accepted cause of cervical cancer. The major oncogenic viral protein, HPV E6, acts by binding and sequestering p53. BARD1 was identified as a binding partner of E6 (Yim et al., 2007). BARD1 expression repressed E6 action in cervical cancer cells, suggesting that BARD1 has tumor suppressor functions and BARD1 deficiency might promote HPV-induced cervical carcinogenesis.

Another role in tumorigenesis might be based on the interaction of the BARD1 C-terminus with the N-terminus of the Ewing's sarcoma (EWS) gene product and the transforming oncogenic fusion protein EWS-FLI1 *in vitro* and *in vivo* (Spahn et al., 2002). Whether binding to BARD1 affects EWS-FLI1 transforming capacity has not yet been investigated.

BARD1 interacts with the transcription factor NF-κB (Dechend et al., 1999). A C-terminal fragment of BARD1, spanning the ANK through the BRCT domains binds *in vitro* to the ANK repeats domain of Bcl-3, a NF-κB inhibitor. Incorrect regulation of NF-κB has been linked to cancer and inflammatory and autoimmune diseases, and might involve BARD1.

Recently, BARD1 emerged as the key player in poly(ADP-ribose) (PAR) signaling after DNA damage (Li and Yu, 2013). Protein PARylation is a first step in DNA double strand break repair. The BARD1 BRCT domains bind to PAR and thus recruit the BARD1-BRCA1 complex to DNA damage sites. This pathway is particularly interesting because of the promising anti-cancer drugs acting on inhibiting the PAR polymerizing enzyme PARP1, which seem more efficient in cells with BRCA1 mutations.

## 6. Genetic and epigenetic modifications of BARD1 in cancer

It is nearly 20 years that the *BRCA1* and *BRCA2* genes were discovered as breast and ovarian cancer predisposition genes (Miki et al., 1994; Wooster et al., 1994). Tweny to 25 percent of familial breast cancer cases are associated with causative genetic modifications, in *BRCA1* or *BRCA2*. The remaining hereditary breast cancer cases are linked to rare mutations or common single nucleotide polymorphisms (SNPs) in other genes, including *BARD1*.

The strong evidence for *BRCA1* and *BRCA2* as predisposition genes is based on germline mutations in protein coding exons. More than 1700 such variants have been identified in *BRCA1* and 2000 in *BRCA2* [https://research.nhgri.nih.gov/projects/bic]. Mutations in protein coding exons of *BARD1* were significantly less frequent (Fig. 4; Table 2), suggesting that cells with such *BARD1* mutations are non-viable. However, with advances in deep sequencing and whole genome sequencing, an ever-increasing number of polymorphisms and SNPs in the *BARD1* gene associated with various cancers is being identified.

### 6.1. *Breast cancer-associated variations of BARD1*

The first missense mutations (p.Q564H, p.V695L, and p.S761N) identified in the *BARD1* gene localized to the C-terminal part of the protein (Thai et al., 1998) and established *BARD1*'s role as a tumor suppressor.

Several mutations were described within *BARD1* in an Italian cohort of familial breast and ovarian cancers without *BRCA1* and *BRCA2* gene alterations, including three missense mutations, p.K312R, p.C557S, p.N295S, one in-frame deletion of seven amino acid residues, c.1075_1095del21 (p.Leu359_Pro365del), and a c.1579C>G transversion with no amino acid change at position p.A502, indicative of a novel polymorphism (Ghimenti et al., 2002). The mutations p.C557S and c.1075_1095del21 were considered as polymorphisms by Thai et al. (1998). Japanese patients with familial breast cancers negative for *BRCA1* or *BRCA2* germline mutations, revealed six alterations in *BARD1*, comprising four missense mutations (p.S241C, p.R378S, p.N470S, p.V507M), one silent mutation (p.H506H), and one in-frame deletion (c.1075_1095del21) (Ishitobi et al., 2003).

A c.1291A>G polymorphism, which translates into an arginine instead of glutamine (p.Q406R), was identified in three out of ten ovarian cancers (Wu et al., 2006). A genotyping analysis of three non-synonymous SNPs, p.P24S, p.R378S, p.V507M in a case-control study of 507 patients with sporadic breast cancer in Chinese women indicated that the polymorphisms p.P24S and p.R378S in *BARD1* may jointly contribute to the susceptibility of breast cancer (Huo et al., 2007). Neither the p.C557S nor the p.V507M alterations were significantly associated with familial or sporadic breast cancer in the Finnish population (Vahteristo et al., 2006).

The screening of 196 non-BRCA1/2 breast or ovarian cancer families for *BARD1* germline mutations identified eleven intron variants and fifteen exon variants, comprising nine missense mutations, four silent mutations, one in-frame deletion and one frame-shift duplication (c.1935_1954dup; p.E652Vfs*69) causing a premature stop and loss of the second BRCT domain of BARD1 (De Brakeleer et al., 2010). Four alterations, namely p.V85L, c.1203T>C, p.I509T, and p.E652Vfs*69, were novel.

Of all *BARD1* variants, p.C557S, residing between the ANK and BRCT domains of BARD1 is the most studied missense mutation (Fig. 4). There is strong evidence that p.C557S is associated with breast cancer risk (Ghimenti et al., 2002; Karppinen et al., 2004, 2006; Stacey et al., 2006). The risk of breast cancer was specifically increased in double carriers of the *BARD1* p.C557S and the *BRCA2* c.771_775del5 mutations and was considered 3-fold greater than the risk for women with only the *BRCA2* c.771_775del5 allele (Stacey et al., 2006). However, other studies did not show a correlation of the p.C557S variant with breast or ovarian cancer (Gorringe et al., 2008; Jakubowska et al., 2008; Johnatty et al., 2009; Spurdle et al., 2011; Vahteristo et al., 2006), further confirmed by a meta-analysis of 20,000 cases and controls (Ding et al., 2011). These discrepancies might be due to population substructure or might indicate that *BARD1* mutations or variants could be deleterious in combination with other genetic changes.

A more complex analysis of *BARD1* modifications in BRCA1/2-negative high-risk breast and/or ovarian cancer patients from Poland identified 16 different *BARD1* variants, five of which were novel. Three of them were potentially pathogenic, including a protein truncating nonsense mutation (c.1690C>T, p.Q564*), a splice mutation (c.1315-2A>G) resulting in exon 5 skipping, and a silent change (c.1977A>G) which alters several exonic splicing enhancer motifs in exon 10 and results in a transcript lacking exons 2-9 (Ratajska et al., 2012). In a recent study, three BARD1 mutations were identified that alter splicing leading to skipping of exons 5, 8, and 2-9, respectively (Ratajska et al., 2015).

A *BARD1* germline mutation with loss of heterozygosity in the tumor was found in a patient negative for *BRCA1* or *BRCA2* mutations (Sabatier et al., 2010). DNA array expression profile of the cancer showed that it resembled the basal-like and BRCA1-mutated phenotypes.

In a gene-sequencing project, *BRCA1* or *BRCA2* germline mutations were found in 18 percent of ovarian cancers and *BARD1* germline mutations in six percent (Walsh et al., 2011) (Fig. 4). This is a much higher proportion of mutations in *BARD1* than identified in previous studies reporting mutations in protein-coding exons only, suggesting that cancer-associated *BARD1* variations might be

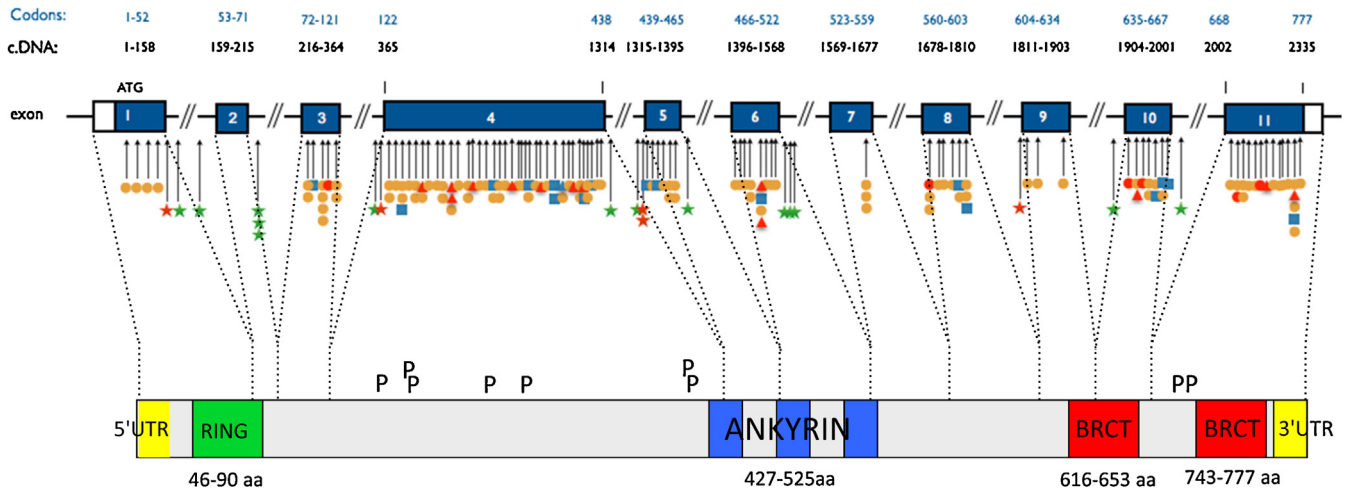


**Fig. 4.** Mutations and polymorphisms of *BARD1*. Intron/exon structure of *BARD1* is shown on the top with corresponding cDNA and amino acid (codons) residues above. The protein structure is shown with phosphorylation sites (P), RING, ANK, and BRCT motifs below. Arrows mark mutations with circles for missense (yellow) and Nonsense (red) mutations. Deletions/duplications are marked as triangles for in-frame (blue) and frame shift (red). Squares mark silent mutations (blue). Red stars indicate splicing mutations and green stars SNPs within introns. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

**Table 2**
*BARD1* mutations and variants.

| Position | Exon | Mutation (CDS) | Mutation (AA) | Mutation ID (COSM) | No. of records | Mutation type | Type of cancer | Splicing prediction result |
|---|---|---|---|---|---|---|---|---|
| 21 | 1 | c.61C>T | p.R21C | COSM399095 | 1 | Sub. missense | Lung | Low probability |
| 85 | 3 | c.253G>A | p.V85M | COSM159329 | 1 | Sub. missense | Breast | Low probability |
| 104 | 3 | c.312G>C | p.M104I | COSM476870 | 1 | Sub. missense | Kidney | Rather low probability |
| 140 | 4 | c.420G>T | p.K140N | COSM256387 | 1 | Sub. missense | Large intestine | Very high probability |
| 162 | 4 | c.484T>G | p.S162A | COSM145130 | 1 | Sub. missense | Haematopoietic and lymphoid tissue | Rather low probability |
| 163 | 4 | c.487G>A | p.V163M | COSM48253 | 1 | Sub. missense | Lung | Rather low probability |
| 174 | 4 | c.521G>T | p.S174I | COSM402165 | 1 | Sub. missense | Lung | Low probability |
| 183 | 4 | c.547G>C | p.V183L | COSM266081 | 1 | Sub. missense | Large intestine | Low probability |
| 192 | 4 | c.575C>T | p.S192F | COSM573326 | 1 | Sub. missense | Large intestine | Rather low probability |
| 249 | 4 | c.747C>T | p.I249I | COSM1129335 | 1 | Sub. synonymous | Prostate | Rather low probability |
| 254 | 4 | c.760A>T | p.I254L | COSM573327 | 1 | Sub. missense | Lung | Low probability |
| 276 | 4 | c. 825_829delAACTG | p.T276fs*7 | COSM238943 | 1 | Del. frameshift | Prostate | Very high probability |
| 287 | 4 | c.859G>T | p.E287* | COSM172763 | 1 | Sub. nonsense | Large intestine | Low probability |
| 344 | 4 | c.1032T>G | p.S344R | COSM720079 | 1 | Sub. missense | Lung | Rather low probability |
| 352 | 4 | c.1056G>A | p.V352V | COSM365762 | 1 | Sub. synonymous | Lung | Rather low probability |
| 411 | 4 | c.1232C>A | p.P411H | COSM720080 | 1 | Sub. missense | Lung | Rather low probability |
| 413 | 4 | c.1237G>A | p.A413T | COSM720081 | 1 | Sub. missense | Lung | High probability |
| 439 | 5 | c.1317C>T | p.G439G | COSM309429 | 1 | Sub. synonymous | Lung | Rather low probability |
| 441 | 5 | c.1323A>G | p.I441M | COSM209927 | 1 | Sub. missense | Large intestine | Rather low probability |
| 452 | 5 | c.1356T>G | p.S452R | COSM141016 | 1 | Sub. missense | Upper aerodigestive tract | Rather low probability |
| 456 | 5 | c.1366G>A | p.V456I | COSM243168 | 1 | Sub. missense | Prostate | Low probability |
| 460 | 5 | c.1378G>A | p.A460T | COSM209926 | 1 | Sub. missense | Large intestine | Rather low probability |
| 489 | 6 | c.1466C>T | p.T489I | COSM225501 | 1 | Sub. missense | NS | High probability |
| 490 | 6 | c.1468A>C | p.T490P | COSM339124 | 1 | Sub. missense | Lung | High probability |
| 491 | 6 | c.1471G>T | p.G491W | COSM720082 | 1 | Sub. missense | Lung | High probability |
| 515 | 6 | c.1543T>G | p.S515A | COSM377218 | 1 | Sub. missense | Lung | Rather low probability |
| 523 | 6 | c.1567G>A | p.V523I | COSM476869 | 1 | Sub. missense | Kidney | Low probability |
| 558 | 7 | c.1674A>T | p.S558S | COSM720083 | 1 | Sub. synonymous | Lung | Rather low probability |
| 576 | 8 | c.1727G>T | p.G576V | COSM400590 | 1 | Sub. missense | Lung | Very high probability |
| 597 | 8 | c.1791T>C | p.Y597Y | COSM1016065 | 1 | Sub. synonymous | Endometrium | Rather low probability |
| 602 | 8 | c.1805G>T | p.S602I | COSM48252 | 1 | Sub. missense | Lung | Very high probability |
| 648 | 10 | c.1942G>T | p.E648* | COSM573328 | 1 | Sub. nonsense | Lung | Very high probability |
| 660 | 10 | c.1980C>G | p.S660R | COSM720085 | 1 | Sub. missense | Lung | Low probability |
| 665 | 10 | c.1995A>G | p.E665E | COSM243167 | 1 | Sub.synonymous | Prostate | Low probability |
| 665 | 10 | c.1995A>T | p.E665D | COSM250848 | 1 | Sub. missense | Liver | Low probability |
| 674 | 11 | c.2021G>T | p.G674V | COSM324796 | 1 | Sub. missense | Lung | High probability |
| 679 | 11 | c.2037G>T | p.L679F | COSM176125 | 1 | Sub. missense | Large intestine | Low probability |
| 680 | 11 | c.2039G>A | p.W680* | COSM270687 | 1 | Sub. nonsense | Large intestine | Very high probability |

more frequent in non-coding sequences than in coding exons. All studies so far concentrated on penetrance of *BARD1* mutations in both sporadic and non-BRCA1/2 familial breast and ovarian cancers. Whether BARD1 aberrations contribute to BRCA1/2-associated breast/ovarian cancer predisposition has not been specifically investigated.

An alternative to the single gene predisposition was reported by Onay and colleagues (Onay et al., 2006), showing a significant interaction between *BARD1*-[Pro24Ser] and XPD-[Lys751Gln] in a polygenic model of SNPs of low penetrance. Such approaches might identify links of *BARD1* SNPs with different pathways.

### 6.2. *BARD1 variations associated with neuroblastoma and other cancers*

Three common nonsynonymous SNPs in *BARD1* coding regions, p.P24S, p.R378S and p.V507M, which were described in breast and/or ovarian cancers, showed statistically significant association with high-risk neuroblastoma (NB) by a SNP-based genome-wide association study (GWAS), suggesting that SNPs in *BARD1* are not only important for breast and ovarian cancer, but also for other cancers (Capasso et al., 2009, 2013; Nguyen le et al., 2011).

Interestingly, the c.C143T (p.P24S) variation might not induce structural changes, but it alters a potential Kozak motif in front of an alternative translation initiation ATG or an ATG used in an alternative open reading frame (ORF) specific for spliced isoform BARD1β (Fig. 5).

Most importantly, the SNPs with highest correlation scores with NB were found in the 5′ untranslated and in intronic regions of *BARD1* (Capasso et al., 2009). The position of these SNPs and their strong linkage disequilibrium suggested that they might be associated with an alternative splicing mechanism (Fig. 5). This hypothesis was confirmed by the demonstration of increased expression of a spliced isoform BARD1β in NB cells carrying the disease-linked SNP allele (Bosse et al., 2012).

Other independent studies on NB in African American patients associated five SNPs in the *BARD1* region with cancer risk (Latorre et al., 2012). *BARD1* SNPs emerged as the most strongly associated with NB in a comparative study of NB risk loci (Capasso et al., 2013). An analysis of several independent studies revealed that 31 pathways and ten genes are involved in NB, of which *BARD1* and *IL3* are the two most important ones (Lee et al., 2014b).

*BARD1* alterations were also found associated with other cancers and diseases. SNPs or deletions were found associated with myeloproliferative neoplasm (Tenedini et al., 2014), oral cancer (Cengiz et al., 2007), colon cancer (Esteban-Jurado et al., 2015), and predisposition to schizophrenia (van Schijndel et al., 2009).

## 7. Differentially spliced BARD1 isoforms in cancer

Alternative splicing is an important mechanism for the generation of multiple varying mRNA and protein isoforms from a single primary transcript, adding to protein diversity and regulation of gene expression (Kornblihtt, 2007). In humans, over 80% of genes are alternatively spliced (Matlin et al., 2005). Furthermore,

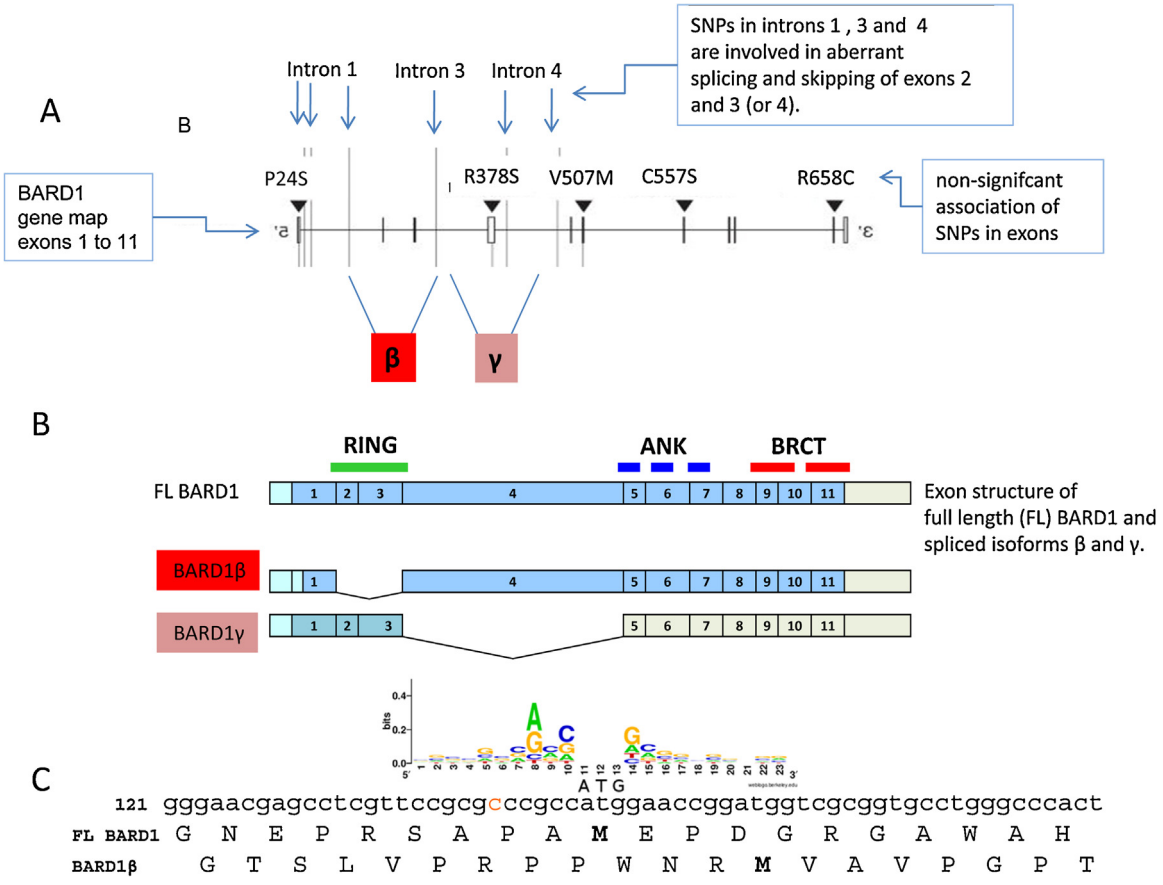


**Fig. 5.** The BARD1 SNPs associated with neuroblastoma. (A) The significant SNPs identified in a GWAS are located in introns (Capasso et al., 2009). SNPs in introns promote alternative splicing, *e.g.* BARD1β or BARD1γ. Mutations in coding regions were not significant. (B) Exon structure of FL BARD1 and of spliced isoforms. (C) DNA sequence of exon 1 is shown with mutation C134T/P24S (yellow) and aligned protein sequences of BARD1 ORF and alternative ORF of BARD1β. The conserved (enlarged) positions of a potential Kosak motif are indicated above. The P24S mutation might diminish translation from the BARD1 ORF and promote translation from alternative ORF. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

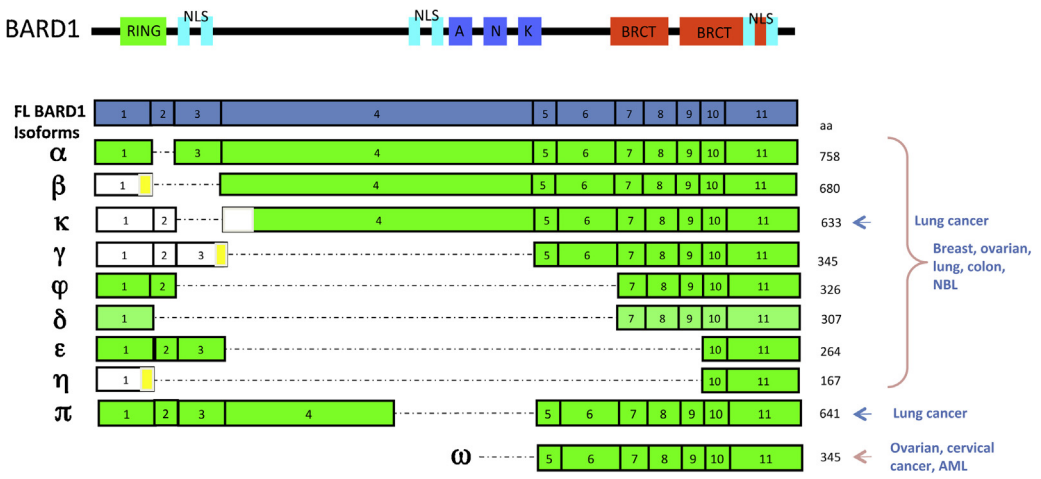


**Fig. 6.** Structure of BARD1 and spliced isoforms. FL BARD1 exon structure (blue) is aligned with spliced BARD1 isoforms below and protein structure above. Spliced variants are named with Greek letters (left) as published for isoforms expressed in gynecological and lung and colon cancers. Presumed protein coding exons of isoforms are shown in green, noncoding exons white, and alternative open reading frames (β, γ and η) in yellow. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

alternative splicing-derived protein isoforms could have aberrant or antagonistic functions. Such variants, in particular BARD1 variants, with dominant negative roles have specifically been found associated with cancer (Chen and Weiss, 2014).

Several splice variants have been identified for BRCA1 (ElShamy and Livingston, 2004; Pettigrew et al., 2010), and *BRCA1* mutations causing splicing were found associated with breast cancer (Thomassen et al., 2012). *BARD1* generates several transcripts by alternative splicing (Fig. 6), which are highly expressed in various cancers (Bosse et al., 2012; Feki et al., 2004, 2005; Lepore et al., 2013; Li et al., 2007a, 2007b; Pilyugin and Irminger-Finger, 2014; Sporn et al., 2011; Wu et al., 2006; Zhang et al., 2011, 2012b). In line with its function as tumor suppressor, *BRCA1* is very frequently methylated in cancer. *BARD1* promoter methylation was found neither in breast (Li et al., 2007b) nor in more than 140 colon cancer cases (Zhang et al., 2012b). These findings suggest that *BRCA1* loss-of-functions, due to mutations or silencing, and aberrant splicing of *BARD1* and gain-of dominant negative functions, is associated with cancer.

Interestingly, BARD1 isoforms were also found highly expressed in human choriocarcinoma and in invasive cytotrophoblasts where their expression is controlled by hypoxia and hormones (Li et al., 2007a).

BARD1 isoforms were detected in different gynecological cancers (Li et al., 2007b; Wu et al., 2006). Cloning of *BARD1* cDNAs from breast, ovarian, endometrial, and cervical cancers showed that a number of protein coding isoforms, termed α, β, κ, γ, δ, φ, ε, η, ω, generated by differential splicing and alternative initiation of transcription, were expressed in all of these cancers, but the relative expression levels of specific isoforms were different in different types of cancer (Fig. 6). BARD1 isoforms were expressed in most breast cancer samples (Lombardi et al., 2007) and correlated with poor prognostics for breast and ovarian cancer (Li et al., 2007b).

BARD1 isoforms first described in gynecological cancers were also identified in non-small-cell-lung cancers (NSCLC) and colorectal cancers (Sporn et al., 2011; Zhang et al., 2012a, 2012b). Interestingly, two novel isoforms, κ and π (Fig. 6), were specifically correlated with the transition from confined to invasive tumors and correlated with poor prognosis and reduced patient survival time in lung and colon cancer (Zhang et al., 2012a, 2012b).

All spliced BARD1 mRNA isoforms were also reported for NB (Bosse et al., 2012). In particular, two NSPs in *BARD1* introns 1 and 3 (Capasso et al., 2009) provided a propensity for differential splicing of the corresponding exons 2 and 3. Indeed, the BARD1β isoform was highly upregulated in NB and in tumor-derived cell lines with the SNP haplotype (Bosse et al., 2012) (Fig. 5).

Interestingly, all BARD1 isoforms lack either the RING finger or ANK repeats, or both, which are required for the tumor suppressor functions (Fig. 6).

### 7.1. *BARD1β, the oncogenic driver*

BARD1 splice variants BARD1β and BARD1δ were first observed in rat spermatocytes and in a rat ovarian cancer cell line NuTu-19 (Feki et al., 2004, 2005). Both isoforms were highly overexpressed together in the NuTu-19 cells, while no FL BARD1 could be detected in these cells neither on the mRNA nor the protein level. NuTu-19 cells are highly tumorigenic and resistant to apoptosis (Feki et al., 2005), suggesting that neither BARD1β nor BARD1δ can substitute for the tumor suppressor functions of FL BARD1.

BARD1β, characterized by lack of exons 2 and 3, translates from an alternative ORF in exon 1 into a protein lacking the RING finger and BRCA1 interaction. Specific selective repression of BRCA1, FL BARD1, or BARD1β, showed that cell proliferation was only slightly affected by FL BARD1 or BRCA1 repression, but repression of FL BARD1 and/or BARD1β lead to a dramatic block of cell proliferation and of cell cycle progression (Bosse et al., 2012; Ryser et al., 2009). Functional studies revealed that BARD1β has a dominant negative function in stabilizing the Aurora kinases A and B (Bosse et al., 2012; Ryser et al., 2009). BARD1β scaffolds Aurora B and BRCA2 at the midbody during telophase and cytokinesis, antagonizing Aurora B ubiquitination and degradation by the FL BARD1-BRCA1 E3 ubiquitin ligase. This results in a similar phenotype as reported for a BRCA2 mutation that affects arrest in cytokinesis (Daniels et al., 2004). Excess BARD1β has a pro-proliferative function by overriding the mitotic checkpoint, as shown *in vitro* for non-transformed fibroblasts, and leads to an invasive phenotype (Bosse et al., 2012). The notion of BARD1β as driver of tumorigenesis is supported by upregulated BARD1β expression associated with NB (Bosse et al., 2012) and poor survival of lung and colon cancer patients (Zhang et al., 2012a, 2012b). A recent study of BARD1 isoform expression in breast cancer reported BARD1α more frequently overexpressed than BARD1β, although not confirmed on the protein level (Wiener et al., 2015).

### 7.2. BARD1δ interferes with estrogen signaling

Another functional isoform involved in tumorigenesis is BARD1δ, characterized by deletion of exons 2-6 which encode most of the RING finger and the ANK repeats. BARD1δ does not interact with BRCA1, whereas it interacts and co-localizes with CstF-50 to cytoplasmic dots in HeLa cells (Tsuzuki et al., 2006). Intracellular localization studies showed that BARD1δ co-localized with mitochondria (Tembe and Henderson, 2007a). Unlike FL BARD1, isoform BARD1δ did not stimulate apoptosis or alter membrane permeability, but might have a function in regulation of mitochondrial response to stress (Tembe and Henderson, 2007a).

BARD1δ mRNA was expressed in all breast, ovarian, uterine, and cervical cancer cell lines, as well as in tissues of gynecological cancers, lung and colon cancer (Li et al., 2007b; Wu et al., 2006; Zhang et al., 2012a, 2012b). It was the only abundantly expressed isoform in cervical cancer and clear cell ovarian carcinoma (Li et al., 2007b).

It was shown that specifically BARD1δ binds ERα and antagonizes FL BARD1 in degradation of ERα, while the BRCA1-BARD1 ubiquitin ligase controls ERα turnover (Dizin and Irminger-Finger, 2010; Eakin et al., 2007). As BRCA1 and BARD1 expression is upregulated by estrogen *via* ERα (Creekmore et al., 2007; Dizin and Irminger-Finger, 2010), this constitutes a regulatory feedback loop. However, estrogen also induces as expression of BARD1 isoforms, including BARD1δ, and as BARD1δ binds to ERα more efficiently than FL BARD1, high levels of BARD1δ will lead to stabilization or to even higher levels of ERα and therefore to increased response to estrogen (Dizin and Irminger-Finger, 2010).

### 7.3. BARD1ω is upregulated in leukemia

A BARD1 isoform containing only exons 6 to 11, BARD1ω, encoding ANK repeats and BRCT domains, is highly upregulated in acute myeloid leukemia (AML) and AML cell lines (Lepore et al., 2013). The specific promoter of BARD1ω has not been identified. Interestingly the expression of BARD1ω is downregulated by HDAC inhibitors (HDACi) that also upregulate microRNAs, comprising miR-19a and miR-19b, which specifically bind to the BARD1 3′UTR.

BARD1ω overexpression induced multiple mitotic defects, including multipolar spindles, but inhibited apoptosis (Lepore et al., 2013). Thus, BARD1ω acts as an oncogene.

## 8. Complex regulation of BARD1 expression

*BARD1* expression is regulated on different levels. On the mRNA level *BARD1* expression is controlled by various signaling pathways (Andre et al., 2015), as well as by cell cycle regulated transcription factors (E2F4) (Ren et al., 2002), and increases during S-phase.

On the protein level, BARD1 protein stability is regulated by stabilizing phosporylation and by degrading and/or cleaving enzymes (Chen et al., 2002; Gautier et al., 2000). BARD1 stability is increased by phosphorylation during the cell cycle, and hyperphosphorylated forms are prevalent during M-phase (Choudhury et al., 2005). Cyclin-dependent kinase 2/cyclin A1 and E1 target BARD1 and affect its stability and heterodimer functions with BRCA1 (Hayami et al., 2005).

While BRCA1 is targeted for degradation by ubiquitination-dependent pathways (Choudhury et al., 2004; Rodriguez et al., 2004; Wu et al., 2010), BARD1 is one of four proteins that are degraded in a cell cycle regulated manner by the anaphase promoting complex (APC/C) (Song and Rape, 2010). Another proteolytic modification of BARD1 occurs during apoptosis, namely cleavage by the calpain protease (Gautier et al., 2000). Thus, BARD1 levels increase during S-phase, due to transcriptional upregulation and increased protein stability, reaching a peak in mitosis.

Another inducer of BARD1 expression is estrogen *via* ERα (Creekmore et al., 2007). The hormone-induced expression of BRCA1 and BARD1 or BARD1 isoforms might provide a link that partially explains the increased risk of breast/ovarian cancer associated with BARD1 or BRCA1 deficiencies and estrogen exposure.

Upregulated expression of BARD1 was also associated with hypoxic conditions and with response to genotoxic cellular stress in different mouse tissues (Irminger-Finger et al., 2001; Jefford et al., 2004; Li et al., 2007a). In tumor tissues, FL BARD1 is underrepresented or not present, while differentially spliced isoforms are over-expressed and associated with carcinogenesis. Understanding the regulation of expression of FL BARD1 and its isoforms is therefore of utmost importance.

The expression of the majority of protein-coding BARD1 isoforms is driven by the *bona fide* promoter located upstream of exon 1 of *BARD1* (Li et al., 2007b). According to the analysis of chromatin modification associated with transcription, this promoter appears to be active in the majority of tissues reported in public databases (Pilyugin and Irminger-Finger, 2014). It was also shown that the expression of *BARD1* is positively regulated by estrogen. However, the estrogen response element (ERE) was identified in intron 9 of *BARD1* (Creekmore et al., 2007; Dizin and Irminger-Finger, 2010). Interestingly, this ERE is located 73 kb downstream of the *bona fide* *BARD1* transcription start site but in the proximity of the transcription start site of a non-coding BARD1 isoform BARD1 9′L (Fig. 7). In healthy individuals, modifications associated with active transcription from the BARD1 9′L promoter were only observed in B lymphocytes and endothelial cells. However, in cancer cell lines and human cancers BARD1 9′L is highly expressed (Pilyugin and Irminger-Finger, 2014).

BARD1 9′L shares 3′end sequences with protein-coding BARD1 mRNAs and could function as competing endogenous RNA (ceRNA), regulating the expression of BARD1 mRNAs (Pilyugin and Irminger-Finger, 2014). ceRNAs represent one type of the recently emerged functional long non-coding RNAs (lncRNA), which share microRNA recognition elements with specific mRNAs and can compete for microRNA binding and thus affect the stability and function of these mRNAs (Panzitt et al., 2007; Poliseno et al., 2010; Salmena et al., 2011).

Available data suggest that the 250 nucleotides after the translation stop codon of the BARD1 3′UTR are a minimal fragment of the BARD1 3′UTR shared by FL BARD1 and shorter oncogenic BARD1 mRNA isoforms (Pilyugin and Irminger-Finger, 2014). However, FL BARD1 has a much longer 3′UTR and may be down-regulated by microRNAs seeding in the region that oncogenic BARD1 isoforms are lacking (Fig. 7). As a result, the ratio of FL BARD1 *versus* isoforms may be shifted in favor of oncogenic BARD1 isoforms, thus driving carcinogenesis. There is accumulating evidence that links the BARD1 3′UTR to microRNAs and cancer. One example for such regulation is BARD1 SNP rs7585356, which is associated with NB (Capasso et al., 2009) and modifies the overlapping target sequence of several microRNAs and potentially affects their binding and the microRNA-dependent regulation of expression of *BARD1* and its isoforms in NB (Pilyugin and Irminger-Finger, 2014).

Furthermore, BARD1 9′L has been shown to compete for microRNA binding in the case of miR-101 and miR-203 *in vitro* (Pilyugin and Irminger-Finger, 2014). Similarly, miR-19a and miR-19b were reported to down-regulate the expression of the cancer-associated BARD1ω isoform in acute myeloid leukemia and might be inhibited by a ceRNA-dependent regulation of BARD1ω expression (Lepore et al., 2013).

Changes in the epigenetic and hormone-dependent gene regulation accompanying carcinogenesis might activate the expression of BARD1 9′L in cancer cells, thus promoting stability of oncogenic BARD1 isoforms.

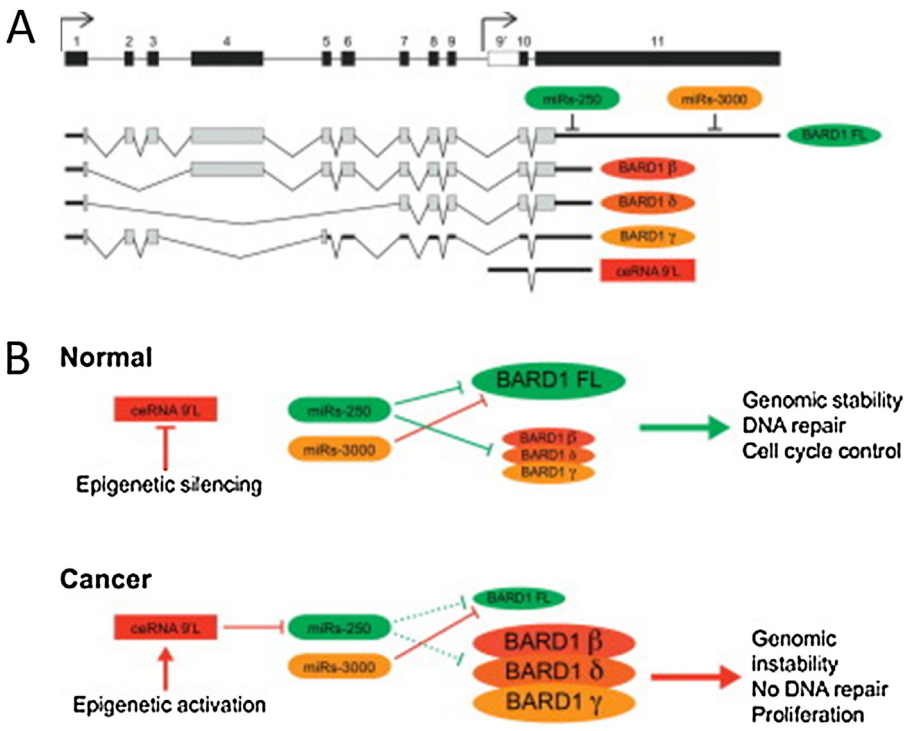


**Fig. 7.** Regulation of FL *BARD1* and isoforms by microRNAs and BARD1 9′L. (A) Genome structure and 3′UTR region of FL BARD1 is shown with arrows indicating positions of transcription start sites from common promoter of FL BARD1 and isoforms and BARD1 9′L promoter. A scheme of cDNA structures of FL BARD1 and isoforms, including BARD1 9′L (ceRNA 9′L), is presented with respective 3′UTRs and the microRNAs targeting different regions of the UTRs: miRs-250, binding to the 250 bases after the stop codon, miRs-3000, binding to the longer 3′UTR of FL BARD1. (B) Model for microRNA and BARD19′L regulation of FL BARD1 and isoform expression. Combinations of microRNAs targeting 3′UTR positions 1–250 (miRs-250) or positions 251–3000 (miRs-3000), are expressed in a tissue-specific manner and effect repression of FL BARD1 as well as isoforms. FL BARD1 contains a long 3′UTR (positions 1–3000), but isoforms tend to have shorter 3′UTRs (BARD1β, BARD1γ, and BARD1δ). In healthy tissues (Normal) microRNAs maintain an equilibrium of FL BARD1 and isoforms in favor of FL BARD1 required for genomic instability, DNA repair, and cell cycle control functions. In cancer cells, the BARD1 9′L promoter is active, BARD1 9′L competes for binding of miR-250s, but not miR-3000s, thus creating a disequilibrium in favor of BARD1 isoforms with a short 3′UTR. As BARD1 isoforms antagonize FL BARD1 functions, this leads to genetic instability, loss of DNA repair and cell cycle control functions, and permits uncontrolled proliferation.

## 9. Therapeutic possibilities

BARD1 isoforms are highly upregulated in cancer cells antagonizing the functions of FL BARD1. In normal cells FL BARD1 has a function in turnover of the mitotic Aurora kinases, which are essential for proper cell division. Overexpression of Aurora kinases is observed in many cancers and is associated with genetic instability and aneuploidy, which makes the Aurora kinases emerging drug targets (Marzo and Naval, 2013; Wang et al., 2009). The switching from FL BARD1 to BARD1β permits to override cell cycle blocks due to the deregulated turnover of the Aurora kinases. Thus, Aurora and BARD1β expression levels might be predictive biomarkers for response to Aurora inhibitors.

Estrogen is the major risk factor for breast and ovarian cancer and anti-estrogen preventive measurements are proposed for genetically predisposed women. Estrogen acts *via* ERα in activation of transcription and cell cycle progression. The caveat exists that most breast cancers with BRCA1 mutations are ER negative. It is noteworthy that in ERα positive cells BARD1δ is upregulated in response to estrogen, but in ER negative cells, BARD1δ is expressed constitutively (Dizin and Irminger-Finger, 2010). This might be explained by the presence of a repressor, which is activated in ER positive cells, but lost in ER negative cells. This repressor could be a BARD1 3′UTR binding microRNA, or the competing ceRNA BARD1 9′L. Inhibiting BARD1 isoform expression or/and BARD1 9′L might be a novel pathway for therapeutic intervention or/and prevention for predisposed women.

PARP inhibitors (PARPi) are the group of pharmacological inhibitors of the enzyme poly ADP ribose (PAR) polymerase (PARP) used for the treatment of cancer. Upon DNA damage, PARP proteins recruit DNA repair proteins, in particular BRCA1 and BARD1. Therefore, PARPi cause multiple double strand breaks, and in tumors with BRCA1 or BRCA2 mutations these double strand breaks are even less efficiently repaired, leading to more rapid cell death (Lee et al., 2014a).

The BRCA field was focused on investigating mostly the role of BRCA1, BRCA2, and less so of BARD1, in DNA recombination and DNA repair pathways. However, the finding that BARD1 binds to PAR and guides PARP to the side of DNA repair (Li and Yu, 2013), brings BARD1 in the center of mechanisms that are important for understanding the PARPi-mediated therapies. To determine how the expression of BARD1 isoforms contributes to the success of PARPi therapy will be crucial, as BARD1 isoforms are highly expressed in BRCA1 mutation-linked tumors (Wu et al., 2006).

## 10. Co-evolution of BRCA1, BARD1, and BARD1 isoforms?

As pointed out above, BARD1 and BRCA1 share homologies in the N and C-terminus comprising the RING motif and the BRCT domains, but no homology is found for the middle regions. The N and C-termini of both proteins are not only homologous, but also have conserved intron–exon junctions in different species (Irminger-Finger and Leung, 2002). While these similarities suggest a common evolutionary ancestor for *BARD1* and *BRCA1*, there are additional factors that indicate a separate, but converging, evolution.

Protein sequence alignment of *BARD1* translation products of different species shows that the BARD1 N-terminus is less well conserved than the C-terminus (Fig. 2). BARD1 protein sequences from different species show little or no homology within the regions

upstream of the RING finger motif. In many species, protein translation starts at the second methionine of the human sequence, and in some cases the translation start-encoding exon 1 has no relationship with human exon 1 (*e.g. BARD1 canis lupus* exons 1 is not related to human BARD1), or the RING finger motif is degenerated (*BARD1 arabidopsis*). From this one might conclude that a BARD1 protein existed as an ANK and BRCT containing protein and evolved and acquired the RING finger.

Interestingly, all BARD1 isoforms lack either RING or ANK motifs or both, but not the BRCT domains. Furthermore, BARD1 isoforms are not unique to cancer cells. Indeed, BARD1 isoforms are highly expressed in human cytotrophoblasts and are associated with cytotrophoblast invasion and proliferation (Li et al., 2007a). Whether isoforms are essential for successful cytotrophoblast invasion remains to be determined. One might hypothesize that isoforms comprising the BRCT domains, or BRCT and ANK, existed as separate genes, and exon shuffling during evolution generated a gene coding for RING, ANK, and BRCT domains, with tumor suppressor functions. Different evolutionary trees are indeed found for the BRCT domains of BARD1 and BRCA1 (Sheng et al., 2011).

It is possible that BRCT containing truncated forms of BARD1 are archaic forms of BARD1. As it is established that BRCA1 needs BARD1 for stability, intracellular localization, and tumor suppressor functions, one might ask: Does BARD1 need BRCA1? This remains to be elucidated.

## Acknowledgements

We would like to thank our colleagues, Drs. Pierre-Alain André, YongQiang Zhang, and Lin Li, for discussions and insight in unpublished data. This work was partially supported by grants of the Swiss National Science Foundation (Grant No. 31003A-141018) to IIF.

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